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Development of five digits is controlled by a bipartite long-range cis-regulator

Laura A. Lettice, Iain Williamson, Paul S. Devenney, Fiona Kilanowski, Julia Dorin and Robert E. Hill*

ABSTRACT

Conservation within intergenic DNA often highlights regulatory elements that control gene expression from a long range. How conservation within a single element relates to regulatory information and how internal composition relates to function is unknown. Here, we examine the structural features of the highly conserved ZRS (also called MFCS1) cis-regulator responsible for the spatiotemporal control of Shh in the limb bud. By systematically dissecting the ZRS, both in transgenic assays and within the endogenous locus, we show that the ZRS is, in effect, composed of two distinct domains of activity: one domain directs spatiotemporal activity but functions predominantly from a short range, whereas a second domain is required to promote long-range activity. We show further that these two domains encode activities that are highly integrated and that the second domain is crucial in promoting the chromosomal conformational changes correlated with gene activity. During limb bud development, these activities encoded by the ZRS are interpreted differently by the fore limbs and the hind limbs; in the absence of the second domain there is no Shh activity in the fore limb, and in the hind limb low levels of Shh lead to a variant digit pattern ranging from two to four digits. Hence, in the embryo, the second domain stabilises the developmental programme providing a buffer for SHH morphogen activity and this ensures that five digits form in both sets of limbs.

KEY WORDS: Sonic hedgehog, Long-range regulation, ZRS, Limb development, Mouse

INTRODUCTION

Multi-species conserved non-coding elements occur in the vertebrate genome and are clustered often within large gene deserts in the vicinity of developmentally regulated genes (Boffelli et al., 2004; Woolfe et al., 2005). Many act as cis-regulators of transcription and may reside at long distances from the genes they regulate (Visel et al., 2009). A highly conserved 780-bp element called the ZRS is an example of this class of cis-regulator. ZRS sits in an intron of the ubiquitously expressed Lmbr1 gene and from here it operates over a distance of 1 Mb of DNA to control the precise spatiotemporal expression of the Shh gene in both the fore and hind limbs (Lettice et al., 2002, 2003; Sagai et al., 2005). Shh is a morphogen that is contained within a 1.7-kb HinDIII fragment (Lettice et al., 2008) (Fig. 1A). Constructs containing this element express the lacZ reporter gene in the mesenchyme at the posterior margin of the limb bud in transgenic mice, reflecting the endogenous Shh pattern (Fig. 1B,C). The expression activity is, however, confined to the highly conserved ~780-bp fragment (Fig. 1A,D,E) (Lettice et al., 2012) (the ratio of expressing to total transgenic embryos for each construct is listed in Table 1). In order to dissect the ZRS further, a series of terminal deletions from the 3′ end of the 1.7-kb fragment were made (orientation of the ZRS defined relative to the 5′ end of Shh) (Fig. 1A) and analysed for spatial patterns of expression in transgenic embryos harvested at approximately embryonic day (E) 11.5. The constructs called DelA and DelB deleted 232 bp and an additional 74 bp (Fig. 1A),
respectively; both showed expression at appreciable levels in the posterior margin of the limb bud (Fig. 1F-I). We previously showed that members of the ETS transcription factor family function at the ZRS to establish and restrict the boundary of Shh expression to the posterior margin of the limb (Lettice et al., 2012). GABPα and ETS1 bind to multiple sites, in particular two high affinity sites (sites 1 and 3 shown on Fig. 1A) to regulate the position of the expression boundary and at least one of these high affinity sites is required for reporter gene expression. The DelB transgenic construct removed all but site 1 and, accordingly, the lacZ reporter gene was expressed in transgenic embryos (Fig. 1H,I); whereas further modification to specifically mutate the remaining ETS site (the consensus ETS binding site AGGAAATG at site 1 was converted to GCCAAAGT inDelB-ETS, Fig. 1A) (Lettice et al., 2012) showed no detectable expression (Table 1).

Further deletions caused substantial reductions in the spatial expression pattern as exhibited by constructs DelC and DelD (Fig. 1J-M), which removed an additional 41 bp and 98 bp, respectively (Fig. 1A). The DelC construct revealed lower limb expression and the fore limbs were more susceptible than the hind limbs to this loss of sequence (Fig. 1J,K), suggesting that a forelimb regulatory element lies within the 41-bp fragment between the DelB and DelC deletions. However, the 41-bp sequence was specifically deleted from the intact ZRS (Del41; Fig. 1A) and showed no reduction in expression (Fig. 1N,O), compared with DelB, in either the fore or hind limbs. The final terminal deletion (DelD construct in Fig. 1A) caused a complete loss of forelimb expression, a substantial decrease in the hind limb expression (Fig. 1M) and, overall, a reduction in the percentage of expressing embryos (Table 1). The contribution of the 3′ half of the ZRS, using the 3′ END fragment (equivalent to the sequence deleted in DelD in Fig. 1A) was examined but no detectable limb expression (Table 1) was observed, suggesting that this half of the ZRS carries no independent spatial activity. These data indicate that the spatial activity lies in the 5′ half of the ZRS but the activity relies on an accumulative input from throughout the ZRS. Notably, these analyses also showed that the fore and hind limbs respond differently to the loss of sequence information, the fore limbs being more susceptible to this loss.

Fig. 1. Deletion analysis of the ZRS cis-regulator. (A) Diagram of the Shh locus including the upstream gene desert and the position of the ZRS within an intron of the Lmbr1 gene. An expanded view of the 1.7-kb HindIII genomic fragment containing the 780-bp highly conserved ZRS is shown with the position of the point mutations (bars) and the ETS binding sites (green ovals) marked. The deletion constructs used in the transgenic analysis are shown below. (B-O) Expression of the lacZ gene in E11.5 embryonic limb buds (fore limbs in the top row and hind limbs in the bottom panel) for the 1.7-kb wild-type fragment (B,C), the conserved 780-bp ZRS (D,E), the DelA deletion (F,G), the DelB deletion (H,I), the DelC deletion (J,K), the DelD deletion (L,M) and the Del41 deletion (N,O). No expression was observed for the DelB-ETS or the 3′ END construct.
ZRS activity requires integration of sequence information

Although the 3′ END construct directs no detectable transgenic activity, a number of point mutations associated with preaxial polydactyly reside in this region (Fig. 1A) (Anderson et al., 2012). One well-studied polydactylous mouse mutant, called hemimelic extra toes (Hx) (Fig. 2A), is responsible for Shh ectopic expression in limb buds (Blanc et al., 2002; Lettice et al., 2003). Accordingly, mouse transgenics that incorporate the Hx mutation direct expression (Maas and Fallon, 2005; Lettice et al., 2008) that reflects this mutant pattern (Fig. 2B,C). The deletion series reported above removed the domain containing the Hx point mutation but no ectopic expression was detected, indicating that the Hx point change acts as a gain-of-function mutation, perhaps generating a novel factor binding site similar to that shown for the AUS and Family AC mutations (Lettice et al., 2012). If the Hx mutation generates a novel binding site for an as-yet-unidentified factor then, alternatively, mutations designed to disrupt binding at the Hx site specifically should act similarly to the deletions and generate only posterior expression. Nucleotide substitutions at the Hx site that should alter binding, a 5-bp replacement (REP5) and two different 3-bp replacements (REP3A and REP3B) (collectively called the REP mutations) (Fig. 2A), all unexpectedly inactivated the ZRS (Table 1). This local disruption of potential factor binding at the Hx site had more severe consequences than did the terminal deletions, suggesting that overall structural architecture plays a crucial role in this region of the ZRS.

Table 1. Details of transgenic constructs and the number of transgenic embryos obtained

<table>
<thead>
<tr>
<th>Construct</th>
<th>Base changes</th>
<th>Junction fragment</th>
<th>Number stained/ number of TGs</th>
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<tbody>
<tr>
<td>1.7 kb</td>
<td></td>
<td></td>
<td>10/13</td>
</tr>
<tr>
<td>780 bp ZRS</td>
<td></td>
<td></td>
<td>6/10</td>
</tr>
<tr>
<td>DelA</td>
<td></td>
<td></td>
<td>12/17</td>
</tr>
<tr>
<td>DelB</td>
<td></td>
<td></td>
<td>9/12</td>
</tr>
<tr>
<td>DelB-ETS</td>
<td>GTCACCTCCTCTCTT to GTCACCTGAACCTCTT</td>
<td></td>
<td>0/7</td>
</tr>
<tr>
<td>DelC</td>
<td></td>
<td></td>
<td>6/8</td>
</tr>
<tr>
<td>DelD</td>
<td></td>
<td></td>
<td>3/13</td>
</tr>
<tr>
<td>Del41</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3′ END</td>
<td>TTTTTTGGCACTGAT to TTTTTTGGCACCAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rep5</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Rep3B</td>
<td>TTTTTTGGCACCAGT to TTTTTTGGCACCAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLIP80</td>
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<td></td>
<td></td>
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<tr>
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<td></td>
<td></td>
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<tr>
<td>FLIP80+REP3A</td>
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<tr>
<td>Core</td>
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<tr>
<td>M100081+REP3A</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1.7 kb+Shh Prom+β-gal</td>
<td>TTTTTTGGCACCAGT to TTTTTTGGCACCAGT</td>
<td></td>
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</tr>
<tr>
<td>DelB+Shh Prom+β-gal</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Shh Prom+β-gal</td>
<td>TTTTTTGGCACCAGT to TTTTTTGGCACCAGT</td>
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<td></td>
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</table>

Base pair changes are shown in bold.

We predict that the REP mutations disrupt factor binding such that context-dependent protein interactions are altered leading to local inactivation of this region. Terminal deletions, by removing all the regulatory information, bypass these local structural changes such that activity becomes solely dependent on information encoded in the 5′ half of the ZRS. To investigate these local interactions further, the relative position of the Hx and REP mutations within the ZRS were rearranged. A gain-of-function Hx mutation should be context independent such that the Hx mutation site placed anywhere within the ZRS would show ectopic activity. The REP mutations, by contrast, should be highly dependent on the relative position in the sequence. A series of internal inversions were made to test these predictions. An 80-bp highly conserved region was inverted within the context of the intact ZRS such that the encoded information of the inverted 80 bp is retained but situated in the opposite orientation. This construct was called the Flip80 construct (Fig. 2A). Transgenic embryos carrying Flip80 exhibited expression along the wild-type posterior margin (Fig. 2D,E). By contrast, ectopic, anterior expression was detected (Fig. 2F,G) in Flip80 carrying the Hx mutation (Flip80+Hx, Fig. 2A) showing that the Hx-mediated ectopic expression is not context dependent, consistent with Hx acting as a dominant gain-of-function mutation. Next, we made the inversion construct carrying the inactivating REP3A mutation, called Flip80+REP3A (Fig. 2A). Transgenic embryos carrying the REP3A mutation in this context, rather than inactivating the ZRS, showed restoration of expression in the posterior margin (Fig. 2H,I). To assess
whether at the inversion breakpoints crucial factor binding sites may have been disrupted, we made another series of inversion constructs (Fig. 2A) called Flip49 and Flip49+REP3A. Both of these exhibited solely posterior expression of the reporter gene (Fig. 2J-M), similar to the Flip80 series. Hence, we suggest that the ZRS activity is not simply a clustering of binding sites but has a structural component key to its activity. Accordingly, the terminal deletions appear to remove sufficient sequence at the 3′ end such that only the spatial information of the ZRS (residing mostly at the 5′ end) is available in the transgenic assay. The REP mutations, by contrast, cause local structural perturbations that affect the whole of the ZRS acting in the manner of an enhanceosome (Panne, 2008). The enhanceosome model suggests that the DNA-protein complex affects structural architecture essential for regulatory activity and, in accordance, the REP mutations underscore the role that local structural integrity plays in affecting the overall activity of the ZRS regulator.

**The point mutations re-direct spatial activity**

Because the gain-of-function Hx mutation lies within the 3′ half of the ZRS, we examined the ability of this mutation to impart

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**Fig. 2. Mutational analysis within the ZRS.**

(A) The modified ZRS constructs used in transgenic analyses. The positions of the point mutations are marked by bars and M100081 and Hx highlighted in green and red, respectively. The sequence changes made within the Hx domain are also shown in red. (B-Q) Expression of the lacZ gene in E11.5 embryonic limb buds (fore limbs in the top row of each and hind limbs below) for the construct carrying the Hx mutation (B,C) (data from Lettice et al., 2008), the Flip80 construct (D,E), the Flip80+Hx construct (F,G), the Flip80+REP3A construct (H,I), the Flip49 construct (J,K), the Flip49+REP3A (L,M), the Core fragment (N,O) and the Core fragment carrying the M100081 mutation (P,Q). No expression was observed for the 3 END+Hx, the REP3A, REP3B, REP5 or the M100081+REP3A constructs.
autonomous ectopic activity. The Hx point change was incorporated into a construct containing the 3′ END fragment (3′ END+Hx, Fig. 2A). The transgenic embryos showed no expression at any site in the embryo (Table 1), suggesting dependence on further information, presumably from the 5′ domain of the ZRS.

To examine further the sequence requirements for driving ectopic expression, we focused on the mouse M100081 mutation (Masuya et al., 2006). The minimal sequence tested that efficiently directed spatial expression in the limb pattern is the Core 443-bp fragment (Fig. 2N,O); created by deleting within the conserved sequence of the ZRS 24 bp from the 5′ end of DelB (Fig. 2A). The M100081 mutation was added to the Core element (Core+M100081, Fig. 2A) and expression was detected, as expected, in the posterior margin but the mutation redirected the expression domain to the anterior of the limb bud (Fig. 2P,Q). To further show that the ectopic expression is dependent on the posterior spatial activity, we added the mutation to the intact ZRS [transgenic expression previously shown by Lettice et al. (Lettice et al., 2008)] and the ZRS carrying the inactivating REP3 mutation (M100081+REP3A). In the resulting M100081+REP3A transgenic embryos, both the posterior expression and the ectopic activity were undetectable (Table 1). Both the Hx and M100081 polydactylous mutations required the spatial information encoded in the 5′ half of the ZRS to operate and, hence, the point mutations function by redirecting expression to the additional, ectopic site in the limb bud. This further supports the notion that the information in the 3′ half is conveyed along the ZRS to affect spatial transcriptional activity encoded in the 5′ half.
3’ end of the ZRS required for long-range activity

Transgenic assays gauge regulatory activity of enhancers outside the normal chromosomal context and in close proximity to a promoter. In order to understand the full regulatory capacity, the ZRS was assayed in its native context by targeting mutations to this locus in embryonic stem cells (ESCs) to modify the endogenous genomic sequence. The targeted alleles were designed to include the lacZ reporter gene inserted downstream to monitor the regulatory activity of the mutated ZRS from a short range (Fig. 3A). Three targeting constructs were made (supplementary material Fig. S1A,B). Firstly, a construct that deleted the 3’ end fragment (region deleted in the DelB transgenic) was targeted and the mutant mice were designated ZRS3 del+LACZ/+ (Fig. 3A). The second construct (Fig. 3A) was similar but with the single remaining ETS1/GABP binding site intact and this line was designated ZRS3 del+LACZ+/+. The third was the control construct (Fig. 3A) in which the ZRS was left intact but the reporter gene was placed downstream and this line was designated ZRS wt+LACZ+/+.

Initially, the lacZ expression pattern (Fig. 3B-E) was analysed in the different mouse lines. Two different mouse lines carrying the ZRS3 del+LACZ+-targeted locus (designated ZRS3 del+LACZ20 and ZRS3 del+LACZ232) were generated to ensure that any phenotype detected was due to the targeted mutation, and subsequent analysis on both lines showed no detectable differences in the limb bud phenotype. (In those instances in which no specific line number is stated, the line shown is ZRS3 del+LACZ20.) Expression in ZRS3 del+LACZ20/+ was compared with that of the ZRS wt+LACZ12 line (shown in Fig. 3B-E). Both spatial and temporal expression were accurately regulated in the ZRS3 del+LACZ+/- lines compared with ZRS wt+LACZ/+, initiating expression at the appropriate stage, ~E10.5 (Fig. 3B,C), in both fore and hind limb and continuing to E11.5 (Fig. 3D,E). Expression of lacZ regulated by ZRS3 del+LACZ/+ did, however, show a decrease in width compared with ZRS wt+LACZ (compare Fig. 2E with 2D) presumably due to the differences in number of ETS binding sites (Lettice et al., 2012). Levels of lacZ mRNA in both the ZRS3 del+LACZ+/- lines were compared with the ZRS wt+LACZ+/- line by qRT-PCR (Fig. 3H). Limbs from 11 ZRS wt+LACZ12, eight ZRS3 del+LACZ20/- and 14 ZRS3 del+LACZ232/- were analysed (Fig. 3H). The short-range activity was further analysed by in situ hybridisation. As the selection cassette used to make the ESC lines also contains a PGK-neo<sup>R</sup> gene, probes to both neo<sup>R</sup> and lacZ were used and showed no detectable differences in expression levels (Fig. 3J-M). These analyses showed that levels of expression, monitoring short-range transcriptional activity, in the limb bud are similar whether the ZRS is intact or is lacking a short-range activity, in the ZRS wt+LACZ(+/-) embryos, but, by contrast, consistently showed limb deformities. The forelimbs were severely affected, presenting with severe truncations similar to the Shh loss-of-function alleles (Chiang et al., 2001; Sagai et al., 2005) (data not shown). The hind limbs, however, presented with a unique series of abnormalities and severe distal truncations in both sets of limbs (Fig. 3J). These limb defects were presumably due to regulatory interference by the cassette containing the lacZ and neomycin resistance (neo<sup>R</sup>) gene (Fig. 3A). This regulatory interference was eliminated by the recombination of the surrounding loxp sites (Fig. 4A) in both the ZRS3 del+LACZ+/- and ZRS wt+LACZ+/- mice and these lines were designated ZRS3 del+/- and ZRS wt+/-, respectively (Fig. 4A).

The ZRS wt+/- and the ZRS3 del+/- mice were backcrossed to make homozygous mutant mice. The ZRS wt+/- embryos had normal fore and hind limbs (Fig. 4B,E). The ZRS3 del+/- embryos, by contrast, consistently showed limb deformities. The forelimbs were severely affected, presenting with severe truncations similar to the Shh loss-of-function alleles (Chiang et al., 2001; Sagai et al., 2005) (data not shown). The hind limbs, however, presented with a unique series of digits forming in an incomplete pattern (Fig. 4F′-F′, Table 2). The range of hind limb phenotypes included thickening, shortening and often fusions of the tibia and fibula (Fig. 4C,D), loss or fusion of the tarsals, loss of metatarsals often showing fusions and digits ranging from two to four (Fig. 4F′ ). The ZRS3 del crossed to the Shh null mouse (Shh<sup>null</sup>) results in ZRS3 del+/-;Shh<sup>null</sup>/+ embryos with a similar range of limb deficiency phenotypes (Table 2). This genetic non-complementation confirms that the ZRS deletion mutation is operating through the Shh gene.

The ZRS 3’ deletion is compromised at the level of normal long-range distance regulation, raising questions about the ability of the deletion mutations to recognise the Shh promoter. We argue that both the ZRS and the 3’ deletion mutation are promiscuous in their enhancer activity showing a capacity to recognise heterologous promoters (the β-globin promoter driving β-gal and the PGK promoters driving Neo<sup>R</sup> in Fig. 3J-M). To examine specifically whether the ZRS 3’ deletion could recognise the Shh promoter, we made constructs containing the Shh promoter linked to the lacZ gene. This promoter construct contains the conserved 375 bp from the translational start codon extending 5’ to a non-conserved region of simple repeat. The expression of this construct, controlled by either the full-length 1.7 kb or the DelB fragment, was examined and both are capable of driving specific fore and hind limb expression (Fig. 3N,O) to a similar efficiency (Table 1). The construct in the absence of an added enhancer showed no consistent embryonic expression (Table 1). The 3’ domain of the ZRS does not appear to be required for recognition of the Shh promoter.

At E11.5, by in situ hybridisation, we show that Shh is normally expressed at the posterior margin of the limb (Fig. 4G,H) and Ptc (PtcΔ1 – Mouse Genome Informatics), a sensitive readout for Shh activity (Marigo et al., 1996), is expressed deep into the middle of the limb bud (Fig. 4K,L). In the ZRS3 del+/- embryonic forelimbs at E11.5, the expression of both Shh (Fig. 4I) and Ptc (Fig. 4M) is undetectable. In the hind limbs, expression of Shh is low but detectable (Fig. 4J) and Ptc is considerably reduced (Fig. 4N). At E10.5, when Shh and Ptc are first detectable in both fore (Fig. 4O,S) and hind limbs (Fig. 4P,T), neither are detected in either set of limb buds in the ZRS3 del+/- embryo (Fig. 4Q,R,U,V), suggesting that there is a lag in the emergence of SHH activity. Expression levels of Shh were then analysed on pairs of limb buds from individual embryos at E11.5 by qRT-PCR and the mean of the individual pairs plotted (Fig. 4W; individual pairs of limbs from five wild-type and six mutant embryos were analysed). In forelimbs, the levels of Shh mRNA was very low (<0.03% that of the wild type). In the hind limb, the levels were ~10% of wild-type levels (Fig. 4W) showing that a surprisingly low level of Shh expression is necessary to initiate digit number specification. To establish a range in which Shh
functions, we analysed levels of Shh expression in the heterozygous Shh\textsuperscript{null/+} mouse, which has normal legs (individual limb bud pairs from 13 wild type and 14 heterozygotes were analysed). As expected, Shh expression in limb buds from the Shh\textsuperscript{null/+} embryos was about half of wild-type Shh levels (55%, Fig. 4W) revealing that a dynamic range of Shh levels act to regulate digit number. Hence, in the hind limbs, digit 1 forms in the absence of Shh expression (Chiang et al., 2001), whereas at low levels (~10% of wild-type
levels), up to four well-formed digits develop and then by approximately half the normal levels the limbs have the wild-type complement. Therefore, formation of five digits in the hind limbs occurs at a Shh level between 10 and 55% of wild type, indicating that a significant buffering capacity exists for producing digits and that the upper limit for digit formation is set at five.

### 3′ deletion of the ZRS mediates chromosomal conformational changes

Amano et al. (2009) showed that the competence to express Shh in the limb bud is due to long-range enhancer-promoter interactions. To analyse changes to long-range chromosomal conformation due to the 3′ deletion of the ZRS enhancer, we used 3D-FISH (fluorescence in situ hybridisation to chromosomal DNA) (Chambeyron and Bickmore, 2004; Morey et al., 2007; van de Corput et al., 2012) using individual fosmid probes encompassing the ZRS and the Shh gene (supplementary material Table S2) or a cloned probe carrying the lacZ/Neo selection cassette. We chose to examine samples at E11.5 as in situ data shows that Shh is expressed in the hind limbs of ZRS3′del/3′del at this stage, making it possible to compare active (in ZRS3′del/3′del) and inactive (in ZRSwt/lacZ) conformations at the same stage. Thus, E11.5 day embryos were sectioned and the region of the limb that expresses Shh (the ZPA) was determined by immunohistochemistry using an anti-Shh antibody raised against the non-signalling C-terminal portion of the protein (Fig. 5A,B). Alternate sections were used to highlight the Shh-expressing regions for FISH analysis.

Initially, the heterozygous E11.5 embryos carrying the ZRSwt/lacZ allele were examined. In the cells analysed, we were able to distinguish the wild-type allele from the insertion allele (carrying the selection cassette and lacZ) using probes for Shh, the ZRS and lacZ/PGK-Neo (Fig. 5C,D). Distances were measured in cells in expressing (ZPA) and non-expressing (limb tissue proximal to the ZPA) regions of the limb and the distribution in 0.2-μm intervals was plotted (Fig. 5E). In the non-expressing tissue, the distance between the ZRS and the Shh gene was similar for each allele, particularly at the closest interval (<0.2 μm). In the ZPA, the conformation of the wild-type allele (WT F-ZPA, Fig. 5E) showed significantly closer association between the ZRS and the Shh gene than in non-expressing cells, in agreement with a previous report (Amano et al., 2009). In the insertion allele, however, the association was not significantly different from that in non-expressing cells (Fig. 5E), suggesting that although the ZRS is active at close range to drive lacZ expression, the insertion element inhibited long-range interactions. The insertion carries two basal promoters and presumably successfully competes for the ZRS enhancer activity. (P-values for the statistical analyses can be found in supplementary material Tables S3-S6.)

We next looked at the intact ZRS (ZRSwt) and the ZRS 3′ deletion (ZRS3′del) alleles (Fig. 5F,G) in which the selection cassettes (lacZ and PGK-neo) had been removed. The ZRSwt allele showed a significant difference in ZRS and Shh colocalisation in expressing and non-expressing regions of the limb and was similar to the unmodified wild-type allele. The ZRS3′del, by contrast, showed no differences in the forelimb (Fig. 5H). Here, the comparison was between proximal non-expressing tissue and distal posterior cells in which Shh would normally be expected to be expressed. In the hind limbs, expressing tissue is still identifiable by the Shh antibody. Interestingly, measurements show that in the ZRS3′del/3′del a mean of 27% of the nuclei show close proximity (<0.2 μm), significantly different from 39% determined for the ZRSwt (P=0.04) (Fig. 5I,J). In comparison with non-expressing tissue, which shows a mean of 22.5%, there may be a difference which correlates with the low levels of expression in the ZRS3′del/3′del, however, this association is not significant (Fig. 5J). (Tables of P-values can be found in supplementary material Tables S3-S6.) Thus, there is a correlation between expression levels of the Shh gene and the conformation of the regulatory domain. Deletion of the 3′ end of ZRS results in a reduction in the ability of the ZRS to undergo the chromosomal confirmation associated with gene activity.

### DISCUSSION

Insights into structure-function relationships of cis-regulatory elements are particularly important for understanding how development is programmed. We showed that the conserved DNA sequence that constitutes the ZRS highlights essential interacting regulatory elements. The ZRS sequence can be dissected in transgenic assays into two domains: an active spatial domain that resides within the 5′ half and an apparently dispensable 3′ half that shows no independent spatial activity. However, transgenic analysis supports the notion that the seemingly superfluous sequence encodes information that influences the spatial activity and that integration of information incorporated throughout the ZRS is responsible for full activity. These data fit an enhancerosome model in which the ZRS is organised into different regulatory components that act as a cooperative unit (Panne, 2008).

The function of the 3′ region of the ZRS became obvious after making the mutations at the endogenous locus. Comparison of the intact and 3′-deleted ZRS showed that both are capable of regulating spatial expression at similar levels from a close range but the 3′ end sequence is required to facilitate long-range activity. This inability to drive Shh expression efficiently is not due to lack of recognition of the gene-specific promoter. Although the ZRS3′del allele is inactive at a long range in the fore limbs, in the hind limbs there is low but detectable expression of Shh, indicating a reduction in long-range activity. An element dedicated to long-range activity has been identified in Drosophila (Swanson et al., 2010); we suggest, here, that there is no specific element within the ZRS but rather that long-range activity is due to supplementary regulatory information that boosts the regulatory response. Enhancers are often considered to be binary switches turning genes on and off (Fiering et al., 2000); but, clearly, in the case of the ZRS, an additional set of regulatory instructions is required to meet the challenge of regulating from a distance.

A number of models for how distal regulatory elements communicate with their target promoters have been suggested and the simplest model with the most supporting evidence is of a direct physical interaction brought about by chromatin looping. We used 3D-FISH to examine the colocalisation of the ZRS and the Shh levels, up to four well-formed digits develop and then by approximately half the normal levels the limbs have the wild-type complement. Therefore, formation of five digits in the hind limbs occurs at a Shh level between 10 and 55% of wild type, indicating that a significant buffering capacity exists for producing digits and that the upper limit for digit formation is set at five.

### Table 2. Range of hind limb digit phenotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Five digits</th>
<th>Four digits</th>
<th>Four sets phalanges/three metatarsals</th>
<th>Three digits</th>
<th>Three sets phalanges/two metatarsals</th>
<th>Total number of hind limbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZRSwt</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZRS3′del/3′del</td>
<td>23%</td>
<td>27%</td>
<td></td>
<td>41%</td>
<td>9%</td>
<td>18</td>
</tr>
<tr>
<td>ZRS3′del/3′del/ Shhnull</td>
<td>40%</td>
<td>30%</td>
<td></td>
<td>10%</td>
<td>20%</td>
<td>22</td>
</tr>
<tr>
<td></td>
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</table>

DEVELOPMENT

promoter and showed that the reduction in long-range activity mediated by the deletion of the 3′ half of the ZRS is reflected in the chromosomal conformation. We found a strong correlation between ZRS-promoter colocalisation and expression activity in the limbs in the ZRS alleles containing the interfering selection cassette (ZRSwt+LACZ and ZRS3′del+LACZ) and the allele with the 3′ end deletion (ZRS3′del). Our data suggest a direct relationship between Shh gene expression in the limb bud and chromosomal conformation. As a consequence, we argue that at E11.5 the inactivity of the ZRS3′del in the forelimb and low level of activity in the hind limb is due to an inability to generate the conformation required for gene activity. However, this may be embryonic stage specific. A report (Amano et al., 2009) analysing...
earlier staged embryos (E10.5) showed that in mice with a complete deletion of the ZRS the presence of the enhancer was not required for the interaction of this region with the Shh gene. Therefore, a complex picture is developing in which the ZRS at the earliest stages of limb bud development is driven to interact with the Shh gene by as-yet-identified elements, but this task is subsequently acquired by the ZRS itself by E11.5. We suggest that the 3′ half of the ZRS operates by boosting activity within this mechanism that mediates these enhancer-promoter interactions.

The attenuated expression produced by the ZRS 3′ end deletion revealed an unexpected variability inherent in regulating digit number. The variation in the limb phenotype ranged from a Shh null phenotype in the fore limbs to two to four digits with deletions and fusions of tarsals and metatarsals in the hind limbs. Two parameters of expression that are predicted to be important for SHH morphogen function, i.e. levels and temporal extent of exposure (Yang et al., 1997; Ahn and Joyner, 2004; Harfe et al., 2004), were both affected by the deletion. In addition, the fore limbs showed a greater sensitivity than the hind limbs to regulatory perturbations created by the deletion. The fore and hind limbs are serial homologues that were derived in evolution by duplication from a single set of primordial paired appendages (Shubin et al., 1997). Although fore limbs are structurally distinct from hind limbs in all tetrapods, the pairs are number of defining skeletal characteristics, one of which is the basic number of digits that form. The early developmental programme that establishes the Shh expression in the ZPA to specify digit number is integrated into both sets of limbs. The regulation of this programme, as shown here, does not operate equivalently in the fore and the hind limbs. The 3′ end of the ZRS provides a robust long-range response that ensures that SHH signalling levels are sufficient in both sets of limb buds to promote normal limb patterning.

Buffering of developmental processes against environmental perturbations or mutations was described as canalisation (Waddington, 1942), which has evolved in order to stabilise a phenotype (Gibson and Wagner, 2000). This was shown for some Drosophila regulators; for example, the eve stripe 2 enhancer (Ludwig et al., 2011). The minimal stripe 2 enhancer (480 bp) produces viable offspring; however, surrounding conserved sequences (encompassing 500 bp) are required to buffer against genetic and environmental perturbations. Here, a region of the ZRS provides robustness to Shh limb expression, which ensures an invariant phenotype during limb development, a process that provides developmental stability (Jammiczkzy et al., 2010). High sequence conservation of the ZRS in vertebrates (Lettice et al., 2003) and the limb pattern based on a limit of five digits (Long and Gordon, 2004) is recurrent in land-based tetrapods, suggesting that the phenotype was stabilised in tetrapod evolution and is fundamental to the basic structure of vertebrate limbs. We argue that the robustness assays here evolved to overcome the intrinsic problems of a long-range regulator and provides a mechanism that adds stability to the limb phenotype in tetrapods.

MATERIALS AND METHODS

Transgenic constructs

The full-length ZRS transgenic construct was previously described (Lettice et al., 2003), whereas the inserts for the other constructs were generated by PCR and subcloned into the p1230 vector (Yee and Rigby, 1993) containing the lacZ reporter gene and a β-globin minimal promoter. The Shh promoter constructs were made by cloning the 1.7-kb or DelB ZRS fragment upstream of a Shh promoter that had been generated by PCR. (Primer sequences are in supplementary material Table S1.)

Production of constructs for ESC gene targeting

A 7-kb genomic fragment was retrieved from PAC 542-N10 from the RCP121 library (Osoegawa et al., 2000), corresponding to nucleotides 29, 636, 979 to 29, 643, 992 from mouse chromosome 5 of assembly NCBIM37 (www.ensembl.org). A mini-targeting vector was constructed, based on PL452 (Liu et al., 2003) and the whole construct assembled by recombinengineering (Liu et al., 2003). Details of the constructs and resulting alleles are shown in supplementary material Fig. S1. E14Tg2a ESCs were targeted and each line was screened using primer sets 1f and 1r and 2f and 2r (supplementary material Fig. S1). Correctly targeted ESC clones were microinjected into C57BL/6J embryos to make chimaeras (Dorin et al., 1992). Tetraploid complementation embryos were produced by electrofusion (Nagy et al., 1993) to make entirely ESC-derived embryos. Cre-mediated recombination was performed by crossing to a line carrying a pCAGGS-Cre recombinase gene (Araki et al., 1995).

lacZ expression analysis, skeletal staining and in situ hybridisation

Embryos were analysed for lacZ expression at E10.5 or E11.5 by staining for β-gal activity as previously described (Lettice et al., 2008). Skeletal preparations from E17.5 fetuses were stained simultaneously with Alizarin Red and Alcian Blue (Nagy et al., 2009a,b). Whole-mount in situ hybridisation was performed as previously described (Hecksher-Sorensen et al., 1998).

RNA extraction for qRT-PCR analysis

The limb buds from individual E11.5 embryos were removed and pairs of fore limbs and hind limbs were separately snap frozen, genotyped and RNA extracted using TriReagent (Sigma). Multiplex qPCR assays were performed on a LightCycler480 Real Time PCR System (Roche). Universal ProbeLibrary Reference Gene Assays (Roche) were included such that each sample contained the internal control Gapdh reference gene probe and primers in addition to the target probe (#32 for Shh and #18 for lacZ from the Universal Probe Library) and primers. Each assay was run in triplicate and the mean Cp and the target/reference ratios calculated by the LightCycler480 software (Release 1.5.0). To correct for the variation between litters, the mean wild-type value for each litter was calculated and the individual relative Shh RNA values for both wild type and mutants was divided by the wild-type litter mean. This produces a relative Shh RNA value that is normalised to the wild-type litter mean. These values were subjected to a non-parametric Mann–Whitney U-test and plotted ±s.e.m. using GraphPad Prism 5. Values for lacZ expression were normalised to the levels of expression in ZRSwt.

Mouse embryo sectioning and 3D DNA FISH

E11.5 embryos were fixed in 4% formaldehyde, paraffin embedded and 6-μm thick sections cut. Alternate slides carrying sections through the limb buds were stained with an antibody raised against the C-terminus of SHH (Abcam, ab86462; 1:100) to identify the ZPA and the adjacent slides subjected to FISH. Fosmid clones (supplementary material Table S2) were labelled with digoxigenin-11-dUTP, biotin-16-dUTP or directly labelled with green dUTP (Roche) and were used as probes for FISH. Embryos were analysed for lacZ expression at E10.5 or E11.5 by staining for β-gal activity as previously described (Lettice et al., 2008). Skeletal preparations from E17.5 fetuses were stained simultaneously with Alizarin Red and Alcian Blue (Nagy et al., 2009a,b). Whole-mount in situ hybridisation was performed as previously described (Hecksher-Sorensen et al., 1998).

For 3D analysis of tissue sections, slides were imaged with a Hamamatsu Orca AG CCD camera (Hamamatsu Photonics), Zeiss Axioplan II fluorescence microscope with Plan-neofluor or Plan Apochromat objectives, a Lumen 200 W metal halide light source (Prior Scientific Instruments) and Chroma #89014ET single excitation and emission filters (Chroma Technology Corp.). Images were deconvolved using a calculated PSF with the constrained iterative algorithm of Volocity (PerkinElmer). Image analysis was carried out using the Quantitation module of Volocity (PerkinElmer). For 3D-FISH, 100-175 loci were measured for each tissue and for each probe combination and subjected to Fisher’s exact test.

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Competing interests
The authors declare no competing financial interests.

Author contributions
L.A.L. and R.E.H. analysed and interpreted the data and prepared the manuscript. L.A.L. designed and performed the majority of experiments and I.W. performed and analysed the 3D FISH experiment. P.S.D., F.K. and J.D. contributed to production of the genetic resources and experimental data. R.E.H. supervised the study and wrote the manuscript.

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Supplementary material
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References